the unique *RET* mutations was not possible when the derivative curves overlapped. Although not all pathogenic *RET* mutations were available for analysis, a recent systematic study of high-resolution melting detection of heterozygous point mutations within a PCR amplicon found a sensitivity and specificity of 100% for amplicons <400 bp in size *(15)*. High-resolution melting analysis for mutation scanning is a rapid (1–2 min after PCR), cost-effective assay that requires no processing or separation steps. As applied to *RET* mutation scanning, accuracy of heterozygote detection appears to be 100%, and some (but not all) sequence variations can be distinguished from each other. Because samples are immediately available for further processing after high-resolution melting analysis, the detected variant samples can be sequenced for confirmation of genotype.

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Detection of Biological Threat Agents by Real-Time PCR: Comparison of Assay Performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler Platforms, *Deanna R. Christensen, Laurie J. Hartman, Bonnie M. Loveless, Melissa S. Frye, Michelle A. Shipley, Deanna L. Bridge, Michelle J. Richards, Rebecca S. Kaplan, Jeffrey Garrison, Carson D. Baldwin, David A. Kulesh, and David A. Norwood*[*] (United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; * address correspondence to this author at: United States Army Medical Research Institute of Infectious Diseases, 1425 Porter St., Fort Detrick, MD 21702; fax 301-619-2492, e-mail david.norwood@amedd.army.mil)

Background: **Rapid detection of biological threat agents is critical for timely therapeutic administration. Fluorogenic PCR provides a rapid, sensitive, and specific tool for molecular identification of these agents. We compared the performance of assays for 7 biological threat agents on the Idaho Technology, Inc. R.A.P.I.D.®, the Roche LightCycler®, and the Cepheid Smart Cycler®.**
Methods: **Real-time PCR primers and dual-labeled fluorogenic probes were designed to detect *Bacillus anthracis*, *Brucella* species, *Clostridium botulinum*, *Coxiella burnetii*, *Francisella tularensis*, *Staphylococcus aureus*, and *Yersinia pestis*. DNA amplification assays were optimized by use of Idaho Technology buffers and**

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14. ABSTRACT
Background: Rapid detection of biological threat agents is critical for timely therapeutic administration. Fluorogenic PCR provides a rapid, sensitive, and specific tool for the molecular identification of these agents. A common chemistry that can be used on a variety of rapid, real-time PCR instruments provides the greatest flexibility for assay utilization. Methods: Real-time PCR primers and dual-labeled fluorogenic probes were designed to detect Bacillus anthracis, Brucella species, Clostridium botulinum, Coxiella burnetii, Francisella tularensis, Staphylococcus aureus, and Yersinia pestis. DNA amplification assays were optimized by using Idaho Technology, Inc. buffers and dNTPs supplemented with Invitrogen Platinum® Taq DNA polymerase, and were subsequently tested for sensitivity and specificity on the Idaho Technology, Inc. R.A.P.I.D.®, the Roche LightCycler®, and the Cepheid Smart Cycler®. Results: Limit of detection experiments indicated that assay performance was comparable among the platforms tested. Exclusivity and inclusivity testing with a general bacterial nucleic acid cross-reactivity panel containing 60 DNAs and agent-specific panels containing nearest neighbors for the organisms of interest indicated that all assays were specific for their intended targets. Conclusion: With minor supplementation, such as the addition of Smart Cycler Additive Reagent to the Idaho Technology buffers, a common chemistry could be used for DNA templates that resulted in similar performance, sensitivity, and specificity on all three platforms.

15. SUBJECT TERMS
methods, real-time PCR, Idaho Technology, R.A.P.I.D., Roche LightCycler, Cepheid Smart Cycler, biological warfare agents

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deoxynucleotide triphosphates supplemented with Invitrogen Platinum® Taq DNA polymerase, and were subsequently tested for sensitivity and specificity on the R.A.P.I.D., the LightCycler, and the Smart Cycler.

Results: **Limit of detection experiments indicated that assay performance was comparable among the platforms tested. Exclusivity and inclusivity testing with a general bacterial nucleic acid cross-reactivity panel containing 60 DNAs and agent-specific panels containing nearest neighbors for the organisms of interest indicated that all assays were specific for their intended targets.**

Conclusion: **With minor supplementation, such as the addition of Smart Cycler Additive Reagent to the Idaho Technology buffers, assays for DNA templates from biological threat agents demonstrated similar performance, sensitivity, and specificity on all 3 platforms.**

Because disease-causing microorganisms can be used as aerobiological weapons, accurate and timely identification of these agents is necessary *(1–4)*. Important agents include *Bacillus anthracis* (anthrax), *Brucella* species (brucellosis), *Clostridium botulinum* (botulism), *Coxiella burnetii* (Q fever), *Francisella tularensis* (tularemia), *Staphylococcus aureus*, and *Yersinia pestis* (plague) *(5)*. Real-time PCR can rapidly detect the presence of nucleic acid markers with small reaction volumes and rapid cycling. Assay chemistry is increasingly important in this identification process because the choices and concentrations of enzymes, buffers, salts, primers, and probes affect assay detection limits *(6–12)*.

Real-time PCR assays for detecting biological warfare agents have been developed on the R.A.P.I.D.® (Idaho Technology, Inc.), LightCycler® (Roche), and Smart Cycler® (Cepheid) platforms and are compatible with various fluorescence-based methods such as TaqMan® *(13, 14)*, hybridization probes *(15, 16)*, molecular beacons *(17–19)*, Scorpion primers *(20)*, LUX™ primers *(21–24)*, AEGIS primers *(25–27)*, and SimpleProbes® (IT Biochem). We investigated whether these assays produce comparable sensitivity and specificity on these rapid cycling instruments.

DNA was obtained from recognized culture collections, commercial vendors, clinics, and the United States Army Medical Research Institute of Infectious Diseases collections (for a detailed presentation of the materials and methods, see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue1). We extracted the DNA with reagents from Molecular Research Center, Inc. (Bactozol™) or Qiagen *(28)* and quantified and checked for quality by measuring the absorbance at 260, 280, and 320 nm. DNA amplification capacity was established with a universal primer set to the 16s rRNA gene *(29)*. We confirmed the presence of high–molecular-weight DNA by running 400 ng of each genomic DNA on 0.7% agarose gels and confirmed the identity of each DNA by sequence analysis of the entire 16s rRNA gene and by comparison with published data.

The real-time PCR assay primer and probe sequences are listed in Table 1 of the online Data Supplement. Target sequences were selected for agent specificity. The specific primer and TaqMan or TaqMan–minor groove binder (MGB) probe sequences were designed with *Primer Express*, Ver. 2.0 for Windows (Applied Biosystems). All primer and probe sequences were analyzed with BLASTN for specificity. All primers were synthesized with standard phosphoramidite chemistry with an ABI 394 DNA/RNA synthesizer (Applied Biosystems). The probes (Applied Biosystems) contained 6-carboxyfluorescein (FAM) at the 5′ end and either 6-carboxytetramethylrhodamine (TAMRA) or a nonfluorescent quencher with the MGB protein at the 3′ end. Primer candidates were used to amplify 1 pg of target template in the presence of SYBR green dye (Invitrogen). We eliminated inefficient and/or dimer-producing primer pairs with melting-curve and agarose-gel analysis. Probe concentrations were standardized by diluting the probes so that fluorescence background was 10–30 with a gain setting of 16 on the R.A.P.I.D. platform, 10–30 with a gain setting of 8 on the LightCycler (Ver. 1.2) with LightCycler Software (Ver. 3.3), or 200–400 on the Smart Cycler. $MgCl_2$ and primer concentrations were optimized sequentially on a single instrument: $MgCl_2$ was optimized in 1 mM increments from 3 to 7 mM, and primers were optimized symmetrically in 0.1 μM increments from 0.1 to 1.0 μM. The combinations exhibiting the earliest crossing threshold and generating the highest endpoint fluorescence (EPF) were used for each assay.

Reactions were carried out in either 20-μL (R.A.P.I.D. and LightCycler) or 25-μL (Smart Cycler) volumes. Each assay contained 1× PCR buffer [50 mM Tris (pH 8.3), 250 μg/mL bovine serum albumin; Idaho Technology, Inc.] and 0.2 mM deoxynucleotide triphosphate mixture (Idaho Technology, Inc.). Platinum® Taq DNA polymerase (Invitrogen; 0.8 U per reaction for R.AP.I.D. and LightCycler or 1.0 U for Smart Cycler) was added. Each Smart Cycler reaction also included 1× SCAR buffer [0.2 mM Tris (pH 8.0), 0.2 mg/mL bovine serum albumin, 150 mM trehalose, and 2 μL/mL Tween 20]. Smart Cycler assays incorporating TaqMan-MGB probes were not supplemented with SCAR buffer. Optimal concentrations of primers, probe, and $MgCl_2$ were added, and the master mixture was distributed to reaction tubes to which 5 μL of control/template DNA was added just before analysis. Thermal cycling conditions were as follows: 1 cycle at 95 °C for 2 min; 45 cycles at 95 °C for 0 s (R.A.P.I.D. and LightCycler) or 1 s (Smart Cycler); and 60 °C for 20 s. Fluorescence readings were taken after each 60 °C step.

We made 10-fold serial dilutions in Tris-EDTA buffer (Roche Molecular Biochemicals) from 10 pg to 1 fg, along with a 50-fg calibrator of measured genomic DNA. The calibrators were run in triplicate on the R.A.P.I.D. and Smart Cycler. The detection limit was established by testing a minimum of 60 replicates at a single concentration. The lowest concentration that produced a positive

Table 1. Assay sensitivities as established with at least 60 replicate tests at the limit of detection.[a]

			R.A.P.I.D.		Smart Cycler	
Assay no.	Positive control	Probe	Sensitivity, fg (GE)	Hits	Sensitivity, fg (GE)	Hits
1	*B. anthracis* Ames BACI008	BAPA3P2A	50 (9)	61/62	50 (9)	61/62
		BAPA2340S-MGB	50 (9)	61/61	50 (9)	62/62
2	*B. anthracis* Ames BACI008	BALEF1P1S	50 (9)	62/62	50 (9)	62/62
3	*B. anthracis* Ames BACI008	BACAPBP2	50 (9)	60/62	50 (9)	60/62
4	*B. anthracis* Ames BACI008	BACAPB4P1S	50 (9)	60/62	50 (9)	60/62
5	*Brucella melitensis* BRUC013	OMP2Ap1799-MGB	100 (30)	61/61	100 (150)	60/60
6	*B. melitensis* BRUC013	OMP2Bp39S-MGB	100 (30)	58/60	100 (30)	59/60
7	*C. botulinum* A CLOS001	CBOTA4P2A	100 (25)	60/62	100 (25)	60/60
8	*C. botulinum* B CLOS023	CBOTBP322F	100 (25)	60/62	100 (25)	60/60
		CBOTBP326-MGB	100 (25)	62/62	100 (25)	60/60
9	*C. botulinum* B CLOS023	CBOTBP376-MGB	100 (25)	61/61	100 (25)	60/60
10	*C. burnetii* COXI001	IS1111-p822S	1 (0.5)	60/60	10 (5)	60/60
		IS1111-p822S-MGB	10 (5)	60/60	10 (5)	60/60
11	*F. tularensis* Schu4 FRAN016	FTTULP1F (Tul4-p809S)	50 (27)	62/62	50 (27)	62/62
		Tul4-P819S-MGB	50 (27)	62/62	50 (27)	58/60
12	*F. tularensis* Schu4 FRAN016	FopA-p765S	50 (27)	62/62	50 (27)	61/62
		FopA-P770S-MGB	50 (27)	62/62	50 (27)	62/62
13	*S. aureus* STAP014	SEA318PF	50 (17)	58/60	50 (17)	60/60
14	*S. aureus* STAP014	SEA882PF-MGB	50 (17)	58/60	50 (17)	59/60
15	*S. aureus* STAP014	SEB330PF	50 (17)	62/62	50 (17)	60/60
16	*S. aureus* STAP014	SEB1417PF	50 (17)	62/62	50 (17)	60/60
17	*S. aureus* STAP014	SEB334PF-MGB	50 (17)	62/62	50 (17)	60/60
18	*S. aureus* STAP014	SEB1435PF-MGB	50 (17)	61/61	50 (17)	60/60
19	*Y. pestis* CO92 YERS023	YPPLAP3F	50 (10)	60/62	50 (10)	62/62
20	*Y. pestis* CO92 YERS023	YPPIMP1R	100 (20)	58/60	100 (20)	62/62
21	*Y. pestis* CO92 YERS023	YPCAF1P1383S-MGB	100 (20)	61/62	100 (20)	60/60

[a] Assay limits of detection were defined as the concentration of genomic DNA that produced a positive result in 97% of the replicates tested (58 of 60, 59 of 61, or 60 of 62 positives, depending on number of replicates tested). Molecular weights and genome-equivalents (GE) are provided.

signal in 97% of runs (58 of 60 positive, 59 of 61 positive, or 60 of 62 positive, depending on the number of replicates tested) was considered the assay limit of detection.

We analyzed a panel of 60 organisms (Table 2 in the online Data Supplement). Panels included threat organisms; nearest genetic neighbors to threat organisms; organisms sharing an environmental niche with a threat organism and thus likely to be found in environmental samples; organisms sharing a clinical niche with a threat organism, particularly respiratory pathogens, opportunists, and typical respiratory flora; and organisms observed repeatedly in clinical and environmental samples. In all cases, 100 pg of genomic DNA was used to determine whether the assays cross-reacted with nucleic acids from other organisms. Inclusivity and exclusivity tests were performed on the R.A.P.I.D., and qualitative results were obtained using R.A.P.I.D. Detector Software Ver. 1.2.14.

For the R.A.P.I.D., each reaction capillary tube was read in channel 1 (F1) at a gain setting of 16, with data analyzed with the LightCycler Data Analysis software (Ver. 3.5.3). Qualitative calls were made using the Detector Software (Ver. 1.2.14). For the LightCycler, each reaction capillary tube was read in channel 1 (F1) at a gain setting of 8, with data analyzed with the LightCycler Data Analysis software (Ver. 3.5.3). Sample curves were analyzed by use of the "Second Derivative Maximum" with the baseline adjustment set to "Arithmetic". For the Smart Cycler, data analysis was performed with the Cepheid Smart Cycler software (Ver. 1.2d or 2.0b). Smart Cycler settings consisted of a primary curve analysis with a manual threshold setting of 10, background subtraction turned on, boxcar average set to 5 cycles, background minimum cycle set to 5, and background maximum cycle set to 45.

Whenever possible, targets occurring in multiple copies within the agent genome were exploited for increased sensitivity. Assays were optimized with a standard protocol developed by the Diagnostic Systems Division at the United States Army Medical Research Institute of Infectious Diseases. All primer combinations giving PCR products smaller than 160 bp were tested for amplification efficiency. All probes designed to be used with the most favorable primer pairs were tested for fluorescent signal production. The optimum probe for each primer pair produced the highest EPF signal at a given template input (1 pg for most agents) and standardized probe background. The final primer and probe pairs and optimized assay conditions are listed in Table 1 in the online Data Supplement. Optimal primer and $MgCl_2$ concentrations

were directly transferable among instruments without a loss in sensitivity. The only difference among the instruments was the need for a higher probe concentration and the addition of SCAR buffer on the Smart Cycler. Exceptions were assays that used MGB probes, for which SCAR buffer had a detrimental effect on assay performance.

For most assays, 97% detection (sometimes called sensitivity) was <100 genomic copies on each instrument (Table 1) and was identical among instruments, with 2 exceptions: the outer membrane protein 2a gene in *Brucella* spp. and the assay for the multiple-copy insertion element (IS1111) of *Coxiella burnetii* were less sensitive on the Smart Cycler (Table 1). Primer sets compatible with both TaqMan and TaqMan-MGB probes produced identical results; however, the fluorescent signals of TaqMan-MGB probes generated more robust EPF signals on the LightCycler and R.A.P.I.D. instruments. Interestingly, TaqMan-MGB probes produced weaker fluorescent signals than TaqMan probes on the Smart Cycler, but sensitivity was not compromised. All assays were inclusive to organisms known to have the gene target of interest and exclusive to organisms lacking the corresponding gene targets.

We conclude that the tested assays have comparable sensitivity and specificity on these rapid cycling instruments. The data provide evidence for the easy transfer of assays from one platform to another. TaqMan-MGB probes are attractive because they contain a nonfluorescent quencher and an MGB protein at the 3′ end. The nonfluorescent quencher is more effective in the quenching of reporter dyes than its TAMRA counterpart on traditional dual-labeled TaqMan probes. The MGB increases the melting temperature of the oligonucleotides *(30, 31)*, allowing the use of shorter probes. Consequently, the TaqMan-MGB probes can be designed to target areas where GC content is low, greatly increasing the genetic regions available for assay development. The addition of SCAR buffer to TaqMan-MGB assays on the Smart Cycler had a negative impact on assay performance, perhaps because the probe binding affinity was diminished by buffer component interaction with the MGB tripeptide. We recommend further investigation of the performance of TaqMan-MGB probes with SCAR buffer on the Smart Cycler.

Further testing of the assays presented here could expand data on specificity and could demonstrate utility in clinical and environmental matrices. Although the data in this study were developed on purified nucleic acids, preliminary data with a subset of these assays (assays no. 1 and 20 in Table 1 in the online Data Supplement) indicate that they should perform well in any matrix, as long as sample processing removes inhibitors of PCR *(28, 32)*. This collection of assays provides a repertoire of molecular diagnostic tools that can serve as a foundation for identifying biologic threat agents on multiple, rapid-cycling, real-time, PCR platforms.

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Effects of Folic Acid Before and After Vitamin B_{12} on Plasma Homocysteine Concentrations in Hemodialysis Patients with Known *MTHFR* Genotypes

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Background: **Treatment with folic acid and vitamin B_{12} appears to be effective in lowering total plasma homocysteine (tHcy) concentrations, but whether vitamin B_{12} alone lowers tHcy in patients with normal vitamin B_{12} status is unknown. The aims of the present study were to explore the effect of individual supplementation with folic acid or vitamin B_{12} on tHcy concentrations in hemodialysis (HD) patients and to compare changes in tHcy concentrations with *MTHFR* genotype.**

Methods: **We recruited 200 HD patients (119 men) from the "Umberto I" Hospital (Frosinone, Italy) and the Dialysis Unit of University Hospital "Tor Vergata". These patients were randomized blindly into 2 groups of 100 each. Unfortunately, during the study, 36 patients in the first group and 16 in the second group died. The first group was treated initially with vitamin B_{12} for 2 months and with folic acid for a following 2 months. The second group was treated initially with folic acid and then with vitamin B_{12}. Samples were drawn before administration of either, after the first and second periods, and again 2 months after treatment.**

Results: **The concentrations of tHcy decreased in both groups after the consecutive vitamin therapies, and the decrease was genotype-dependent. The decrease was greater for the T/T genotype ($P < 0.05$) and was more significant when the treatment was started with folic acid ($P < 0.01$).**

Conclusion: **The alternating vitamin treatment demonstrated for the first time the importance of folate therapy and the secondary contribution of vitamin B_{12} in lowering tHcy in HD patients.**

Homocysteine (Hcy) is a non–protein-forming sulfur amino acid that is synthesized from methionine. Hcy can be either remethylated to methione or catabolized through the transsulfuration pathway to form cysteine *(1)*. Hyperhomocysteinemia has been associated with atherosclerosis and arterial thrombosis *(2)*, and evidence suggests that metabolism of folate, vitamin B_{12}, and Hcy is under genetic control.

In patients undergoing hemodialysis (HD), the rate of mortality from cardiovascular disease is 10- to 20-fold greater than that seen in the general population, even after correction for age, sex, race, and the presence of diabetes *(3)*. Hyperhomocysteinemia is common in HD patients, with >90% of dialysis patients having increased concentrations of Hcy.

Increased plasma total Hcy (tHcy) concentrations result chiefly from genetic defects in the enzymes involved in Hcy metabolism *(4)*. Recently, a common C→T mutation at nucleotide position 677 (C677T) has been identified in the gene coding for methylenetetrahydrofolate reductase (MTHFR), which is involved in the remethylation of Hcy *(5)*. The C677T mutation causes a valine-for-alanine substitution, which decreases MTHFR activity and tends to increase tHcy concentrations in individuals who are homozygous for the T/T genotype *(5)*.

In individuals with healthy renal function, the T/T genotype causes only a 25% increase in tHcy concentration compared with persons with other genotypes *(6)*, but in patients with end-stage renal disease undergoing maintenance dialysis, the T/T genotype causes a 40% to 100% increase in tHcy *(7)*.

Folic acid is vital in humans for several metabolic reactions, including the remethylation pathway. However, clinical studies have shown that folic acid therapy is not very effective in normalizing hyperhomocysteinemia in uremic patients *(8)*. In a study by Kaplan et al. *(9)*, vitamin B_{12} supplementation alone, or in combination with folic acid, decreased tHcy concentrations, but full normalization was not achieved. Dierkes et al. *(10)* reported that supplementation with vitamin B_{12} decreases not only tHcy but also serum folate in patients with